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During this year we examined oxime reactivation of organophosphate modified cholinesterases using both achiral and chiral organophosphate inhibitors. The chiral inhibitors were resolved enantiomers of known absolute stereochemistry. This approach has been combined with site specific mutagenesis of the acyl pocket in the enzyme. Our studies clearly point to the importance of an impacted gorge in reducing the efficiency of the oxime reactivation reaction and show that the oximes attack with distinct orientations. Studies on the crystal structure of mouse AChE in the fasciculin complex, in the uncomplexed state and conjugated with organophosphates continue. Finally, we have initiated studies using cysteine mutagenesis to produce modified cholinesterases with altered catalytic characteristics. These studies are probing the dynamics and geometry of the active center of the enzyme while in solution. All of these studies are directed to converting acetylcholinesterase from a stoichiometric to a catalytic antidote for organophosphate toxicity.

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FOREWORD

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INTRODUCTION

During the project period from March 1998 to March 1999, we completed our analysis of the influence of acyl pocket dimensions on the efficacy of oxime reactivation for organophosphates of varying structure. This work is now being extended to other regions of the active center of the enzyme and to additional oximes. These studies reveal the importance of phosphonyl oxygen association with the oxyanion hole in the oxime reactivation mechanism and point to gorge impaction as being a limiting factor in reactivation efficiency. Collaborative studies have been directed to phosphonyl oximes as intermediate, but potent, inhibitors of AChE. Other collaborative studies on the structure of mammalian AChE continue and resolution of the fasciculin complex with mouse AChE has been carried to 2.45Å resolution. A crystal structure of the uncomplexed enzyme has also been obtained at 2.9Å, and we are well along to obtaining structures for the R_p- and S_p-conjugates with AChE. Finally, we have begun a detailed study of AChE structure using cysteine substitution mutagenesis. The cysteine substituted and modified mutants are characterized by steady state kinetics, inhibitor association, trifluoroacetophenone conjugation and spectroscopy of the fluorescently modified inhibitors. We have found this to be a necessary prelude to the production of conjugated oximes on the enzyme.

BODY

I. Mechanism of Oxime Reactivation of Acetylcholinesterase

The purposes of this study are to understand the mechanistic basis of oxime reactivation of AChE and to develop a potential scavenging mechanism for organophosphate nerve agents that is catalytic rather than stoichiometric. Although many of the mechanistic details of the chemistry of oxime reactivation have been elucidated, reactivation has not been analyzed in terms of the structural template now that we have high resolution structures of AChE .

Since in the presence of oximes the alkylphosphates are no longer hemisubstrates forming a stable acyl enzyme, the AChE-oxime combination should be catalytic rather than stoichiometric in that it effects a turnover of alkylphosphate. If retained in the plasma the combination could serve as a scavenger of the alkylphosphates. This concept parallels the approach of Broomfield and Lockridge (1,2), who have modified residues in the oxyanion hole, which allow the cholinesterases to catalyze the turnover of alkylphosphates. The chief limitation of the Broomfield-Lockridge approach stems from the low rate of reaction of the alkylphosphate with the mutated enzyme, whereas the AChE-oxime combination studied here and by others (3-5) may be limited by the slow rate of oxime reactivation relative to the organophosphate reaction. Our objective is thus directed to enhancing oxime reactivation mechanisms and rates.

Our previous studies have utilized the power of combining chiral chemistry and site-specific mutagenesis to understand the organophosphate reaction, and the oxime studies conducted this past year extend the approach from the inactivation mechanism to reactivation mechanisms. To form the enzyme conjugates we have used three enantiomeric pairs: R_p- and S_p-cycloheptyl methylphosphonothiocholine, R_p- and S_p-3,3 dimethylbutyl methyl-phosphonothiocholine and R_p and S_p-isopropyl methylphosphonothiocholine (fig. 1). Thiocholine is a good leaving group, and the polarity of the compounds assures an appropriate margin of safety for our laboratory studies. The three compounds have been synthesized by Dr. Harvey Berman at SUNY Buffalo (6-8) The cycloheptyl methylphosphonate conjugate is

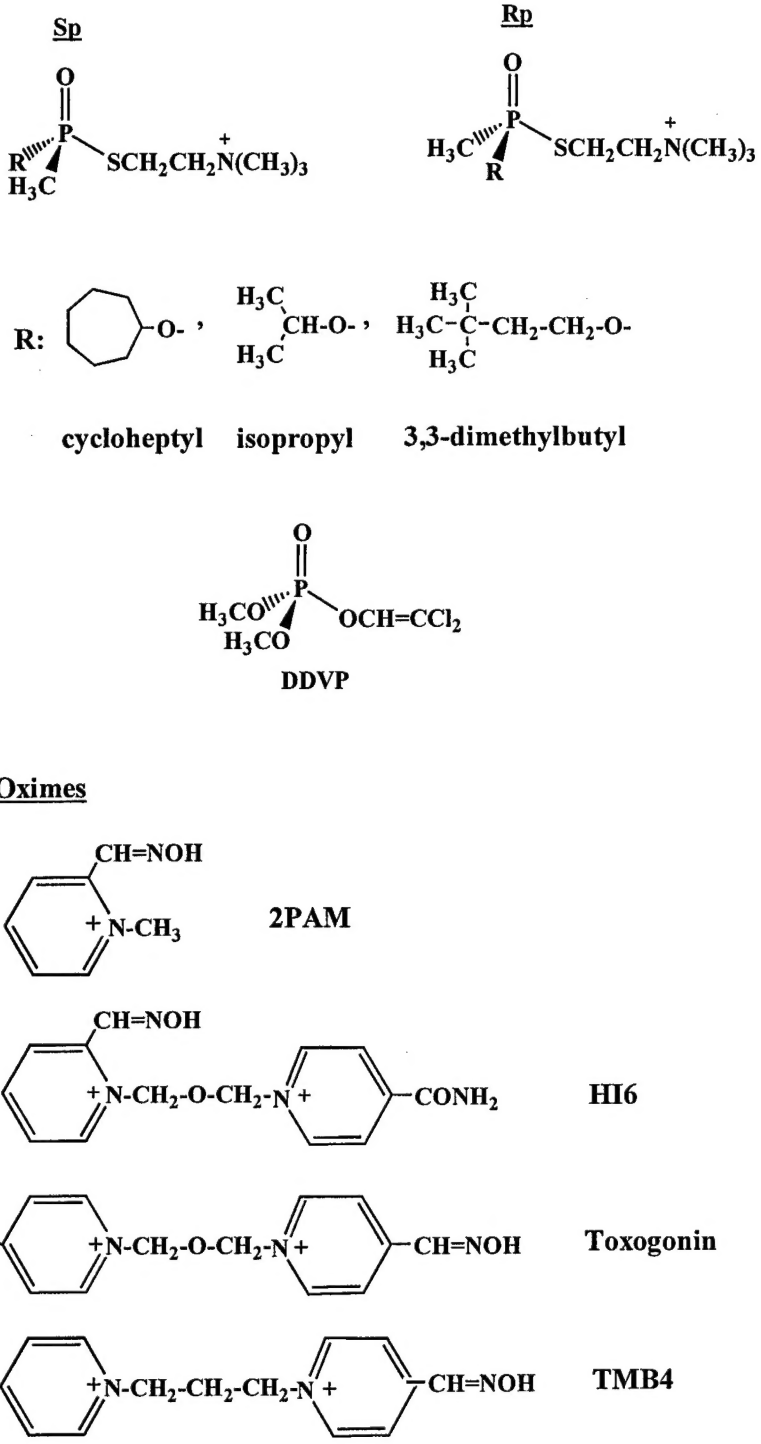
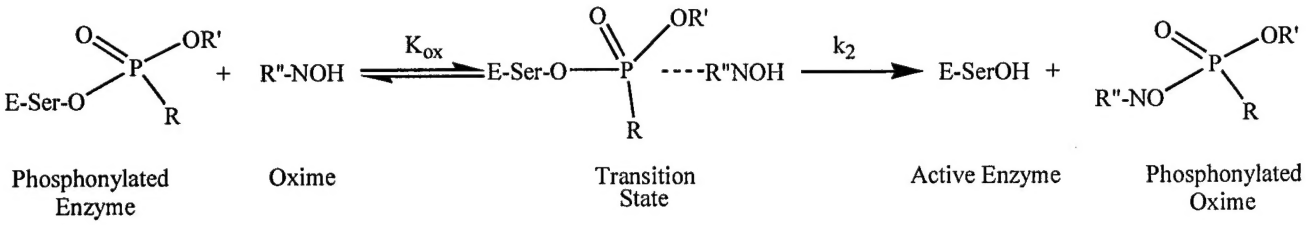


Figure 1 Structures of the organophosphates and oximes used in this study. Upon reaction of the organophosphates with the active site serine, thiocholine and dichloroethylene are the leaving groups forming the respective phospho-serine (203) conjugates on the enzyme.

similar to that formed by cyclosarin, the 3,3-dimethylbutyl methylphosphonate is somewhat similar to soman conjugate, whereas the isopropyl methyl phosphonate conjugate is identical to the sarin conjugate. All six enantiomers will effectively yield diastereomers when reacting with the *l*-serine in the active center of AChE (fig. 2).



Our previous studies showed that a productive phosphonylation reaction required: (a) apposition of the phosphonyl oxygen with the γ-hydroxyl on serine 203; (b) hydrogen bonding between the three hydrogen bond donors, the amide bond hydrogens of Ala204, Gly121 and Gly122, and the phosphonyl oxygen of the inhibitor; and (c) an orientation of the leaving group directed out of the gorge exit. Chiral preferences arise since acyl pocket dimensions limit the R_p phosphonates from achieving this orientation, whereas for the S_p compounds, only the small methyl group needs to be accommodated in the acyl pocket (9-11).

The analyses of the reactivation rates for two oximes, 2-PAM and HI-6, are shown in Tables I-III. Our studies started with an achiral compound, dichlorovinyl dimethyl phosphorate (DDVP or dichlorvos), which forms the dimethyl phosphoryl enzyme conjugate (Table I). Both 2-PAM and HI6 are effective reactivators of the AChE conjugate. The two mutations in the acyl pocket, where the bulky phenylalanines are substituted with aliphatic leucines and isoleucines, F295L and F297I, give rise to no enhancement of oxime reactivation of the dimethylphosphoryl-AChE. In fact, if anything, the reactivation rates are diminished. The reduction in rate may arise from the acquired mobility of the conjugated phosphorate. The F295L mutation results in both a reduction in k_{max} and K_{ox}; the larger reduction in k_{max} gives rise to the overall reduction in the biomolecular rate constant k_r. The influence of the F297I mutation is largely observed on k_{max}. The F295L and F297I mutations correspond to the residues in butyrylcholinesterase and are known to accommodate larger acyl groups in carboxyl ester catalysis (12).

Table II shows the same mutations for HI6 and 2PAM reactivation of three S_p methyl phosphonyl esters with different sizes of the substituted alkoxy group. A comparison for the wild type enzyme shows that both HI6 and 2PAM are most effective in reactivating isopropyl methylphosphonyl-AChE, with HI6 being the more efficient reactivator for all three methyl phosphonyl conjugates. The greater rate of reactivation for the isopropyl substitution suggests that the more bulky alkoxy groups prevent access of the oxime to the phosphorus. Should this be the case, we might expect the steric limitations of an impacted gorge might be alleviated by removal of specific side chains. This is, in fact, borne out by the data which show that the acyl pocket mutations enhance both HI6 and 2PAM reactivation. Of further interest is the surprising observation that HI6 reactivation is enhanced primarily by the F295L mutation and 2PAM is predominantly affected by the F297I mutation. As considered in a subsequent section, this points to distinct angles of attack of the two oximes in the reactivation process.

Table I: Rate Constants for the Reactivation of Mouse Acetylcholinesterase Inhibited with DDVP

2PAM

Enzyme	k_{max} (min^{-1})	K_{ox} (mM)	k_r ($M^{-1}min^{-1}$)	%Reactivation
AChE	0.06	0.28	227	80-100
F295L	0.004	0.04	100	100
F297I	0.02	0.39	51	100

HI6

Enzyme	k_{max} (min^{-1})	K_{ox} (mM)	k_r ($M^{-1}min^{-1}$)	%Reactivation
AChE	0.02	0.19	113	80-100
F295L	0.005	0.09	56	100
F297I	0.01	0.36	28	100

The kinetic constants represent the average or mean of two or more experiments

The interesting observation to emerge from reactivation of the R_p conjugates is their slow rates and in some cases resistance to reactivation (Table III). Neither HI6 nor 2PAM reactivate the R_p cycloheptyl methyl phosphonyl AChE. The R_p isopropyl methylphosphonyl AChE is more slowly reactivated while the S_p and R_p dimethylbutyl methylphosphonyl AChE show roughly comparable reactivation rates. The latter enantiomers contain a primary instead of a secondary alkoxy moiety. Mutation of the acyl pocket to the smaller side chains gives little improvement in reactivation rates of the isopropyl compounds.

It should be noted that the S_p compounds are the most reactive and toxic of the enantiomeric pairs giving rise to the fortuitous and fortunate situation that the more reactive of the enantiomers are also the ones most susceptible to oxime reactivation. This situation is perhaps rationalized in mechanistic terms where insertion of the phosphonyl oxygen in the oxyanion hole is required for both acylation (inactivation by the phosphonate) and deacylation (reactivation by the oxime).

Table IV shows the aging rates for the R_p and S_p enantiomers. For the phosphonate with the primary alkoxy moiety, the 3,3-dimethylbutyl methylphosphonyl conjugate, no aging can be detected over a 72 hour period. As described in a subsequent section, we take advantage of this in forming crystals of the conjugates for structural analysis. For the secondary alkoxy conjugates, cycloheptyl- and isopropyl methylphosphonyl, rates of aging are sufficiently slow as to not interfere with the measurement of reactivation kinetics. For the R_p cycloheptyl and the R_p 3,3-dimethylbutyl methylphosphonyl conjugates, the lack of reactivation precludes an analysis of aging.

Table II: Rate Constants for the Reactivation of Mouse Acetylcholinesterase Inhibited with (Sp) Enantiomeric Organophosphonates

cycloheptyl methylphosphonyl thiocholine

Reactivator	Enantiomer	Enzyme	k_{max} (min^{-1})	K_{ox} (mM)	k_r ($min^{-1}M^{-1}$)	Reactivation(%)**
HI6	Sp	AChE	0.36	2.0	186	96
HI6	Sp	F295L	3.60	1.4	2520	94
HI6	Sp	F297I	--	--	353	127
2PAM	Sp	AChE	0.01	24.0	0.46	70
2PAM	Sp	F295L	0.04	27.0	1.40	76
2PAM	Sp	F297I	0.07	7.5	9.20	74

3,3-dimethylbutyl methylphosphonyl thiocholine

Reactivator	Enantiomer	Enzyme	k_{max} (min^{-1})	K_{ox} (mM)	k_r ($min^{-1}M^{-1}$)	Reactivation(%)**
HI6	Sp	AChE	0.150	0.83	181	97
HI6	Sp	F295L	1.70	1.30	1230	93
HI6	Sp	F297I	0.140	0.44	318	120
2PAM	Sp	AChE	0.042	179	0.24	104
2PAM	Sp	F295L	0.038	35.0	1.10	103
2PAM	Sp	F297I	0.190	6.4	30.0	102

isopropyl methylphosphonyl thiocholine

Reactivator	Enantiomer	Enzyme	k_{max} (min^{-1})	K_{ox} (mM)	k_r ($min^{-1}M^{-1}$)	Reactivation(%)**
HI6	Sp	AChE	0.870	0.56	2160	91
HI6	Sp	F295L	1.050	0.38	3320	105
HI6	Sp	F297I	0.410	0.17	2400	102
2PAM	Sp	AChE	0.130	0.83	155	99
2PAM	Sp	F295L	0.043	0.37	116	107
2PAM	Sp	F297I	0.740	0.95	779	92

The kinetic constants represent the average or mean of two or more experiments

** Extent of reactivation varies slightly with oxime concentration

Table III: Rate Constants for the Reactivation of Mouse Acetylcholinesterase Inhibited with (Rp) Enantiomeric Organophosphonates

cycloheptyl methylphosphonyl thiocholine

Reactivator	Enantiomer	Enzyme	k_{max} (min^{-1})	K_{ox} (mM)	k_r ($min^{-1}M^{-1}$)	Reactivation(%)**
HI6	Rp	AChE	0	0	0	<25
HI6	Rp	F295L	0	0	0	<25
HI6	Rp	F297I	0	0	0	<25
2PAM	Rp	AChE	0	0	0	<25
2PAM	Rp	F295L	0	0	0	<25
2PAM	Rp	F297I	0	0	0.005	92

3,3-dimethylbutyl methylphosphonyl thiocholine

Reactivator	Enantiomer	Enzyme	k_{max} (min^{-1})	K_{ox} (mM)	k_r ($min^{-1}M^{-1}$)	Reactivation(%)**
HI6	Rp	AChE	0.24	1.9	117	86
HI6	Rp	F295L*	0.0007	0.93	0.75	50
HI6	Rp	F297I	0	0	0	<25
2PAM	Rp	AChE	0.0130	28.0	0.52	77
2PAM	Rp	F295L	0.0007	1.0	0.70	68
2PAM	Rp	F297I	0.0008	2.1	0.40	74

isopropyl methylphosphonyl thiocholine

Reactivator	Enantiomer	Enzyme	k_{max} (min^{-1})	K_{ox} (mM)	k_r ($min^{-1}M^{-1}$)	Reactivation(%)**
HI6	Rp	AChE	0.019	0.84	22.0	82
HI6	Rp	F295L	0.001	0.97	1.03	101
HI6	Rp	F297I	0.001	0.97	1.03	83
2PAM	Rp	AChE	0.0120	0.40	36.0	89
2PAM	Rp	F295L	0.0009	0.03	39.0	87
2PAM	Rp	F297I	0.0008	1.03	0.77	134

The kinetic constants represent the average or mean of two or more experiments

* The values for (Rp)F295L only corresponds to the slow phase of reactivation

** Extent of reactivation varies slightly with oxime concentration

To ascertain whether the lack of reactivation for some of the R_p compounds was a consequence of the oxime being *ortho* to the pyridinium moiety and perhaps steric hindrance, we have begun to examine reactivation with additional oximes, toxogonin and TMB-4, where the oxime is situated *para* to the pyridinium nitrogen. These data are shown in Tables V and VI. That complete reactivation of some of the Rp conjugates resistant to HI6 and 2-PAM reactivation can be achieved suggests the absence of aging. Rather accessibility of the oxime to the conjugated phosphorus may be liniting when it resides *ortho* to the pyridinium nitrogen. To examine this question in more depth and detail the efficacy of other oximes for specific phosphonate conjugates, we have started to study reactivation with 3-PAM and 4-PAM. Results

Table IV: Rate Constants for the Aging of Mouse Acetylcholinesterase Inhibited with Respective Organophosphonates

Cycloheptyl methylphosphonyl thiocholine

Enantiomer	Enzyme	k_{aging} (min^{-1})	$t_{1/2}$ (hr)
Sp	AChE	0.00042	24.1
Sp	F295L	0.001	11.6
Sp	F297I	0.0037	30.8
Rp	AChE	NA	NA

3,3-dimethylbutyl methylphosphonyl thiocholine

Enantiomer	Enzyme	k_{aging} (min^{-1})	$t_{1/2}$ (hr)
Sp	AChE	NA	NA
Sp	F295L	NA	NA
Sp	F297I	NA	NA
Rp	AChE	NA	NA

Isopropyl methylphosphonyl thiocholine

Enantiomer	Enzyme	k_{aging} (min^{-1})	$t_{1/2}$ (hr)
Sp	AChE	0.0002	49
p	F295L	NA	NA
Sp	F297I	NA	NA
Rp	AChE	0.00045	26.9

NA = no aging detected over 72 hours

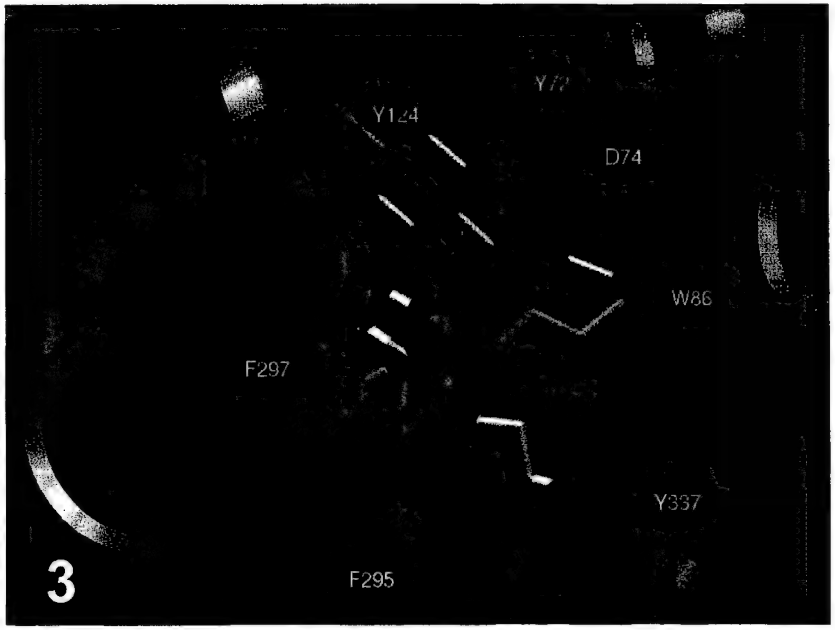
are still to fragmentary to reveal any trends. Nevertheless, these compounds should be forerunners to the more complex TMB-4 and toxogonin structures. The TMB-4 data show diminished stereospecificity for reactivation. However, the data may be complicated by formation of phosphonyl oximes which are also inhibitory to the enzyme.

Figure Legends:

Fig. 3: Starting positions of S_p-cycloheptyl methyl phosphonyl-AChE conjugates for energy minimization in the active center gorge. A starting conformation was built from conformations of congeneric organophosphates in the Cambridge Data Base. The leaving group is removed and the phosphorus is attached to the O-γ-serine with 60° torsional rotations in the P-O-serine bond.

Fig. 4: Energy minimized conformations for S_p and R_p-cycloheptyl methylphosphonyl – AChE. After energy minimization as described in the text for the phosphonates, they were conjugated to the serine with 60° separating each initial configuration (fig. 3). The figure shows the energy minimized conformations obtained and distinguishes the structures with the phosphonyl oxygen out (red) and in (white) the oxyanion hole. Left panel, S_p-conjugate; Right panel, R_p conjugate. In the case of the S_p-stereochemistry, the phosphorus is accessible from the gorge opening.

Fig. 5. Stereoview of the proposed preferred angle of access of 2-PAM and HI-6 to S_p-cycloheptyl methyl phosphonate conjugated AChE. Mutation of F295L enhances the rate of reactivation by HI-6, whereas the F297I mutation enhances 2-PAM reactivation rates. Attack of the phosphorus can be optimized by substitution for the phenylalanines in distinct positions. The orientation shown is from the gorge entry. The phosphonyl oxygen is shown in maroon, and the cycloheptyl and methyl carbons in yellow.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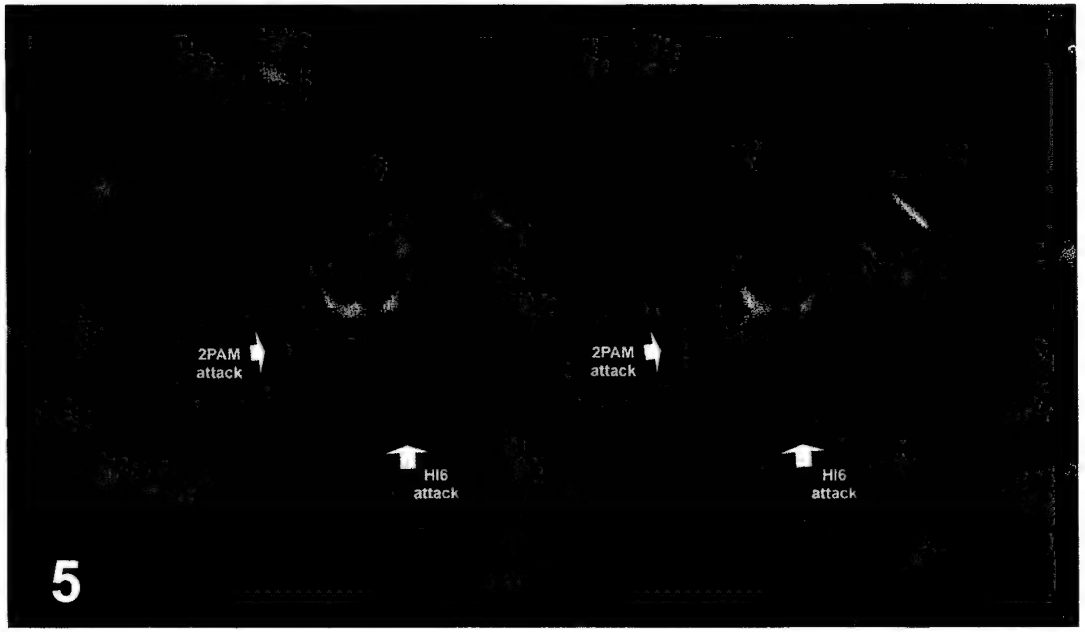
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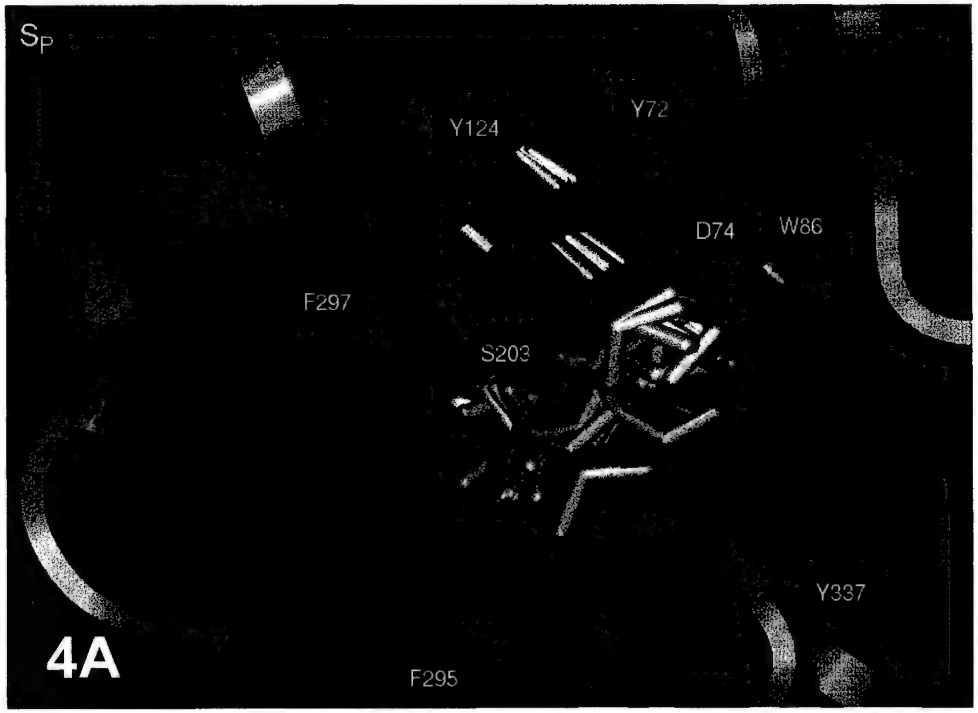
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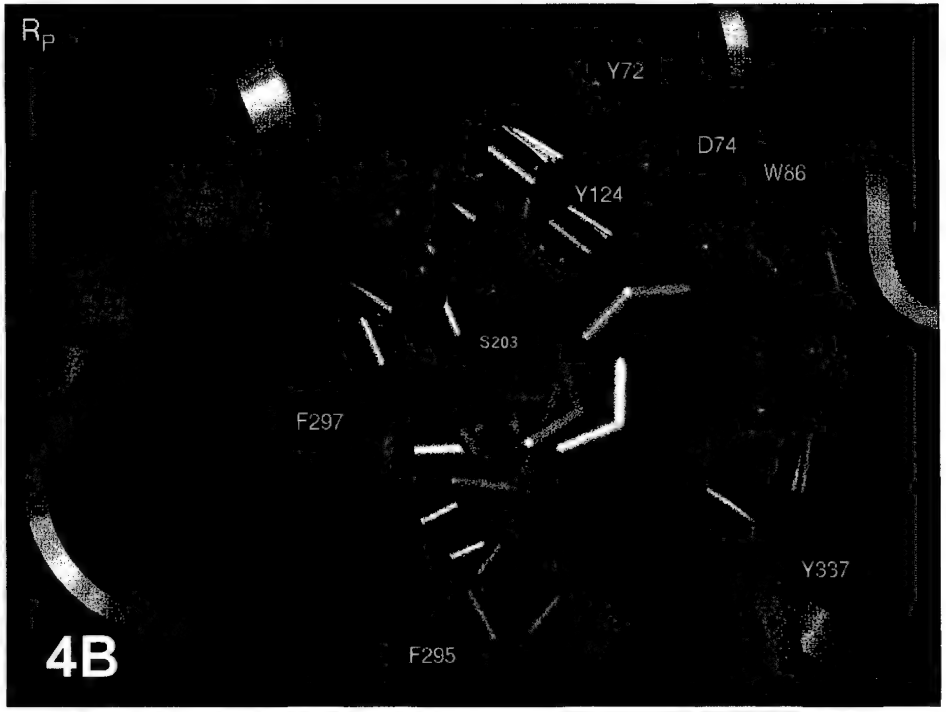
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II. Molecular Modeling of the Conjugates and Oxime Attack Angles Analyzed by Energy Minimization.

A starting configuration of the conjugated phosphonates was built from congeneric organophosphates whose structures were found in the Cambridge Data Base. The AChE-organophosphate conjugate was formed by deletion of the leaving group and placement of the serine γ-oxygen at a covalent bond distance (≤ 3Å) from the phosphate. The structures were then minimized by steepest descent prior to an estimation of the charge distribution. The *ab-initio* approach using a STO-36 yielded the best approximation with the sequence Asp-Ser-Ala and the organophosphate linked to the serine. The amine on the Asp and carboxyl on the Ala were capped as neutral residues.

Using the charges determined from the *ab-initio* calculations, a second minimization was performed where the Cβ-Oγ of the conjugated Ser203 was started in the 6 positions differing by 60° (fig. 3) and side chains from residues 72, 74, 86, 121, 122, 124, 203, 204, 286, 295, 297, 337 and 447 were allowed to move freely. All other residues were fixed. The results of simulated annealing show that for the majority of minimized structures, the phosphonyl oxygen for the S_p enantiomers moves to the oxyanion hole and the cycloheptyl group orients toward the choline site and gorge entrance. For the R_p-enantiomers the bulky group is forced from the acyl pocket to the gorge entrance, diminishing the interactions of the phosphonyl oxygen with the oxyanion hole (fig. 4). Proper positioning of the phosphonyl oxygen depends on it residing within 3Å of the amide backbone hydrogens of Gly 121, Gly 122 and Ala 204. Also, in the case of the R_p enantiomer, the cycloheptyl group resides within the gorge entrance and blocks potential oxime access. These energy minimized conjugates provide a basis for docking of the two oximes (fig. 5).

III. Conclusions and Directions of the Oxime Studies

The availability of a three-dimensional structure of AChE (13,14) and its conjugates (15, 16) enables one to examine reactivity in relation to the geometric confines of the active center. A comparison of reaction rates with enantiomeric phosphonates of known absolute stereochemistry is also of particular value since in the absence of a dissymmetric enzyme active center, the two enantiomers should exhibit identical chemical behavior (11,23). Combining knowledge of AChE structural template and the capacity to alter it through mutagenesis with the enantiomeric selectivity of the phosphonates constitutes a powerful method for delineating orientation of the reactants involved in organophosphate inhibition and reactivation of AChE. Our analysis of the reactivation of the diastereomeric[1] conjugates of R_p and S_p phosphonates with the *l*-serine in the active center reveals several distinctive features of the reactivation reaction.

First, reactivation occurs more readily with the conjugated S_p- than the R_p-enantiomers.The S_p-phosphonates have been shown previously to be the more inhibitory or faster reacting enantiomer (9-11, 17,18). If we assume an orientation, where the leaving group directed out of the gorge and where the phosphonyl oxygen directed towards the oxyanion hole, facilitates the acylation reaction (9-11), then the S_p-enantiomers will have the phosphonyl methyl

[1]linkage to *l*-serine for the diastereomer

directed to the space-confining acyl pocket and the more bulky alkoxyl group will be directed to the choline subsite (fig. 4). With the R_p-isomer, the same constraints on leaving group orientation and on phosphonyl oxygen binding in the oxyanion hole would force the alkoxyl group towards the acyl pocket. Steric limitations in the acyl pocket would relax the positional constraints of the phosphonyl oxygen in the oxyanion hole and the leaving group exiting the gorge (cf: fig. 4). Since oxime reactivation also prefers the S_p-enantiomer, we might assume that orientation of the phosphonyl oxygen in the oxyanion hole facilitates the bond breaking associated with reactivation. Alternatively, distortion of the amino acid side chains necessary for accommodating the R_p-enantiomer in the active center may yield a less reactive species. In either case, the S_p-enantiomer has its attached moieties of differing steric bulk efficiently positioned for acylation and deacylation of the serine by the attacking nucleophile. Maintaining the phosphonyl oxygen in the oxyanion hole is a critical consideration for both reaction mechanisms. However, for the R_p conjugates this forces the bulky alkoxy group into the gorge at a point of narrowing, further precluding access to the oxime (fig.4).

A second general observation stems from the relative resistance to reactivation for the methyl phosphonates substituted with larger alkoxy groups. It was elegantly demonstrated many years ago by Wilson and colleagues that efficiency of the oxime reaction is enhanced by site directing the oxime to the organophosphate through the use of cationic compounds resembling substrates or inhibitors (19,20). This finding takes on considerable practical significance since these oximes are our most efficacious agents in the treatment of organophosphate toxicity through reactivation of inhibited AChE (20). Oximes being relatively strong nucleophiles attack the electrophilic phosphorus breaking the phosphoserine bond and presumably form an unstable oxime phosphonate (fig. 2). More recently, direct conjugates formed between the oxime and phosphonate have been isolated and characterized (21). However, formation of the phosphono-oxime, presumably through a pentavalent intermediate requires direct access of the oxime oxygen to the phosphorus.

Within the confines of the narrow gorge, bulky organophosphates would be expected to enlodge against the gorge wall, thereby limiting access of the oxime to the serine conjugated phosphorus. The enhanced rate of reactivation of the S_p conjugates seen upon substitution for the aromatic groups by the smaller and more flexible aliphatic groups presumably arises from increased oxime accessibility. The influence the acyl pocket mutations also appears to be most dramatic with the larger cycloheptyl and dimethylbutyl moieties with little effect seen for the smaller isopropyl (Table 2) or methoxyl moieties (Table 1). Hence, steric limitations and impaction in the gorge are dominant factors in influencing oxime reactivation rates.

A combined requirement of the phosphonyl oxygen-oxyanion hole orientation and oxime accessibility to the phosphorus in the gorge provides a particular problem for the R_p enantiomers. Orientation of the phosphonyl oxygen to the oxyanion hole forces the alkoxy group to the space confining acyl pocket or to the gorge exit. The former is precluded by the larger alkoxy moieties, while the latter limits access of the oxime to the phosphorus.

A third consideration is based on 2-PAM and HI-6 reactivity being sensitive to the removal of spatial constraints at distinct positions in the acyl pocket. This suggests that the orientation of the two bound oximes and their angles of attack of the phosphorus differ. The

likely attack directions for the two oximes under conditions where the acyl pocket is opened are shown in fig. 5. Since the Phe 297 mutation to a smaller residue enhances 2-PAM reactivation and the Phe 295 mutation affects HI-6 reactivation of the S_p phosphonates, accessibility to the phosphonate from two discrete regions appears to facilitate oxime reactivity. Both reactivators have the attacking oxime situated *ortho* to the pyridinium nitrogen, but the HI-6 structure contains a second pyridinium moiety separated by a flexible 4.5 Å chain. This additional moiety with its cationic group can be expected to enhance affinity; however, it should also constrain the possible orientations of the attacking oxime.

Finally, it is instructive to compare the rates of phosphonate inactivation and reactivation. Even after optimization of the reaction with a more open gorge, reactivation rates by oxime appear relatively slow ($k_r = 10^2$-10^3 M^{-1} min^{-1}) compared to the rate of formation of the phosphonate conjugate with the serine ($k_r \sim 10^8$ M^{-1} min^{-1}). Analysis of the acylation reaction by the phosphonate shows a transition state orientation where the leaving group is directed out of the gorge and the hydrogen bond donors in the oxyanion hole can delocalize the negative charge of the transition state (9-11). This creates near optimal apical positioning of the attacking serine and leaving group in a pentavalent state. By virtue of the geometric constraints of the gorge, the efficiency of the oxime reactivation reaction may be limited by the attacking and leaving group not being in appropriate apical positions for forming an optimal transition state as is the case for acylation by the phosphonate. Hence, even with facilitation of the access of the oxime to the phosphonate, reactivation reactions may have distinct limits to their overall efficiency.

The distinctions in kinetic constants for the series of organophosphates suggest that the rank order of potencies of the oximes in reactivation depend on the structure of the conjugated phosphonate. Thus, efficacies of oximes as antidotes depend on the particular organophosphate used to inhibit the enzyme and agents with larger substitutions may be the least amenable to oxime reactivation. The gorge dimensions create a substantial impediment to oxime access. In circumstances where combinations of oximes and AChE are considered to have catalytic potential in antidote therapy, enhanced reactivation rates and turnover of the conjugated phosphonate can be achieved with certain mutant enzymes that make the active center gorge more accessible.

IV. Crystal Structure of the Mouse Acetylcholinesterase – A Tetrameric Unit Crystal

We are continuing our studies of the structure of mouse AChE. To date, it is the only mammalian AChE structure published (14,28), and virtually all of the mutagenesis studies are being conducted on mammalian enzymes.

Our crystallographic studies are being conducted collaboratively with Drs. Pascale Marchot and Yves Bourne in Marseille, France. The enzyme is prepared in La Jolla, reacted with conjugating phosphonate, shipped to Marseille and the bulk of the crystallography is conducted at the synchroton in Grenoble, France. Recently, we have been successful in obtaining 2.9Å resolution structure in the absence of fasciculin. As described below, a loop from residues 247 to 252 encompassing the second cysteine loop enters the mouth of the gorge of a neighboring subunit giving rise to a tetrameric unit crystal with two of the active centers in the

unit crystal open and two "fitted" with the loop structure from the neighboring subunit. The loop region in other

Table V: Rate Constants for the Reactivation of Mouse Acetylcholinesterase Inhibited with
(Rp)-Organophosphonates by Toxogonin

cycloheptyl methylphosphonyl thiocholine

Enantiomer	Enzyme	k_{max} (min^{-1})	K_{ox} (mM)	k_r ($min^{-1}M^{-1}$)	Reactivation(%)*
Sp	AchE	0.006	0.80	7.40	77
Rp	AchE	0.003	1.02	3.19	65
Rp	F295L	0.003	4.04	0.81	89
Rp	F297I	0.001	1.04	0.90	69

isopropyl methylphosphonyl thiocholine

Enantiomer	Enzyme	k_{max} (min^{-1})	K_{ox} (mM)	k_r ($min^{-1}M^{-1}$)	Reactivation(%)*
Sp	AchE	0.130	2.50	52	90
Rp	AchE	6.01	24.1	249	90
Rp	F295L	0.017	3.03	5.6	97
Rp	F297I	-	-	4.5	84

* Extent of reactivation varies slightly with oxime concentration

Table VI: Rate Constants for the Reactivation of Mouse Acetylcholinesterase Inhibited with Enantiomeric Organophosphonates by TMB4

cycloheptyl methylphosphonyl thiocholine

Enantiomer	Enzyme	k_{max} (min^{-1})	K_{ox} (mM)	k_r ($min^{-1}M^{-1}$)	Reactivation(%)
Sp	AChE	0.174	6.42	27	81
Rp	AChE	0.002	0.05	36	69
Rp	F295L	0.002	0.95	2.11	82
Rp	F297I	0.001	1.01	0.99	65

3,3-dimethylbutyl methylphosphonyl thiocholine

Enantiomer	Enzyme	k_{max} (min^{-1})	K_{ox} (mM)	k_r ($min^{-1}M^{-1}$)	Reactivation(%)
Sp	AChE	0.127	3.23	39	102
Rp	AChE	0.145	2.68	54	82
Rp	F295L	ND	ND	ND	ND
Rp	F297I	ND	ND	ND	ND

isopropyl methylphosphonyl thiocholine

Enantiomer	Enzyme	k_{max} (min^{-1})	K_{ox} (mM)	k_r ($min^{-1}M^{-1}$)	Reactivation(%)
Sp	AChE	2.46	18.4	134	98
Rp	AChE	0.020	0.81	25	77
Rp	F295L	0.015	2.74	5.47	99
Rp	F297I	0.028	21.97	1.27	95

crystals showed a high b-factor. As such, this stretch of sequence was highly mobile and not resolvable in the crystal.

Drs. Bourne and Marchot solved the crystal structure of a monomeric acetylcholinesterase (AChE) from mouse by molecular replacement and refined it to 2.9 Å resolution (R-factor, 0.21) (22). The structure, in which a canonical homodimer assembles between two monomers through a parallel four-helix bundle, reveals a tetrameric assembly of two dimers with antiparallel alignment of the two bundles. In the tetramer, a short Ω loop, bridged by two cysteines and composed of a cluster of hydrophobic residues conserved in mammalian AChEs, along with flanking α-helices, tightly associates with the peripheral anionic site of the facing subunit and sterically occludes the entrance of the gorge leading to the active center. The inverse loop-to-peripheral site interaction occurs within the second pair of subunits; however, the peripheral sites on the two loop-donor subunits remain freely accessible to the solvent. The position and complementarity of the peripheral site-occluding loop mimic the characteristics of the central loop of the peptidic inhibitor, fasciculin, bound to mouse AChE. Tetrameric forms of cholinesterases are widely distributed in nature and predominate in mammalian brain. This structure reveals a likely mode of subunit association and suggests that the peripheral anionic site is a likely site of association of neighboring subunits or heterologous proteins containing surface loops. The structure of mAChE provides significantly improved accuracy in the positions of the main and side chains of the molecule than in the Fas2-mAChE structure, reveals some distinctive features of mAChE compared with TcAChE, and permits direct comparison of a solvent exposed and an occluded peripheral anionic site within the same crystal unit. Also, this structure highlights certain determinants at the surface of the mouse enzyme that could participate in formation of oligomers upon assembly in normal and pathological states, in allosteric modulation of catalysis, or in forming heterologous cell contacts.

Other crystallographic studies are underway in order to improve resolution and define water structure in the crystal. The original fasciculin-complex with mouse AChE (14) now has been refined to 2.45Å resolution structure, a resolution comparable to that reported for *Torpedo* AChE. This mouse structure awaits a final refinement before coordinates are reported.

We have also begun crystallographic studies on the phosphnyl enzyme conjugates, using in this case the S_p and R_p 3,3-dimethyl butylphosphonyl enzyme complexes. Defining the template for oxime attack and perhaps even for a cocrystal with oxime would be very helpful for analyzing the oxime mechanisms described in the initial section.

V. Cysteine Substitution Mutagenesis

These studies are directed to obtaining information on the structure and dynamics of the active center gorge. With this information we hope to tether nucleophiles to the cysteines which may enhance deacylation of the phosphorylated enzyme.

The procedure involves substitution of cysteine for various residues at critical places in the enzyme by classical mutagenesis techniques. The cysteines are then modified by reaction with an electrophile (fig. 6) yielding an artificial side chain, typically by forming a stable thioether linkage. Both the cysteine substituted enzyme and the reaction product are

characterized in terms of catalytic parameters for acetylcholine hydrolysis (K_m, k_{cat}, b and K_{ss}) and for *m*-trimethylammonio trifluoroacetophenone association. In some cases (fig. 7) this can be diagnostic of the extent of reaction with the cysteine. In other cases it is less clear. Fig. 6 shows the various sulfhydryl reactive agents we have employed in this study.

Substitutions on the methane thiorjsulfonate moiety give rise to cationic side chains in the forms of a quaternary ammonium and a primary amine, anionic side chains in the form of a sulfonic acid and neutral side chains containing aliphatic and aromatic residues. These substitutions may alter substrate access through steric bulk or create attractive and repulsive Columbic forces when appropriately positioned with respect to the active center gorge.

Many of our initial cysteiene substitutions did not yield an efficient reaction with the electrophile. This could either arise from a buried cysteine that is inaccessible to the reactant or oxidation of the sulfhydryl forming a disulfide or sulfenic acid. The latter occurred in several of the cases since treatment with dithiothreitol at low concentrations followed by its removal gave rise to a sulfhydryl reactive enzyme. It should be noted that the monomeric form of AChE used in these studies contains six sulfhydryl groups, all of which are paired as disulfides. A spurious reduction will give rise to reactivity of the wide type enzyme. Sulfhydryl substitution allows us to tag the enzyme fluorescently or by radioactivity and the signal difference between the mutant and wide type will independently predict the reactivity of the residue. The fluorescence groups also will yield information on molecular motion (via decay of fluorescence polarization) solvent accessibility (through Stern-Volmer quenching), hydrophobicity or local dipole moment (by Stokes shifts and spectral changes).

These studies look most promising and will be reported in depth in the next progress report. Briefly, to date cysteine has been substituted at 12 different positions and the cysteines have been modified with the thiosulfonates and fluorophores and characterized kinetically. Mutation of Tyr 124 to Cys and modification of the cysteine with acrylodan yields a naphthalene based fluorescent side chain. The acrylodan substitution yields an enzyme still able to bind fasciculin, but with greatly reduced catalytic parameters. DFP reactivity with the active center serine is <2% of the rate with wild type enzyme. Fasciculin binding gives rise to an enhanced acrylodan quantum yield and a hypsochromic shift in the fluorescence emission spectrum consistent with the side chain becoming sandwiched between the aromatic gorge walls and the tip of the fasciculin loop.

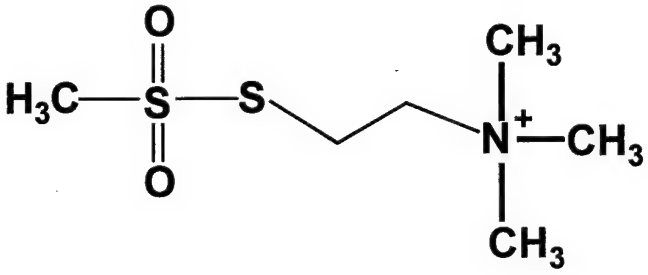
A second modification on substituted Cys 284 on the His enzyme gives rise to a residue modifiable by acrylodan. Its emission spectrum has a longer wavelength maximum consistent with this residue position at an aqueous interface. Formation of a fasciculin complex results in no shift of emission maximum but a slight reduction in quantum yield, consistent with a surface location removed from the active center gorge. In short, the spectroscopic analysis will prove useful in delineating the environment, solvent accessibility and molecular motion of amino acid residues on AChE and these studies will be continued.

Figure 6

Chemical Structures of Labeling Compounds

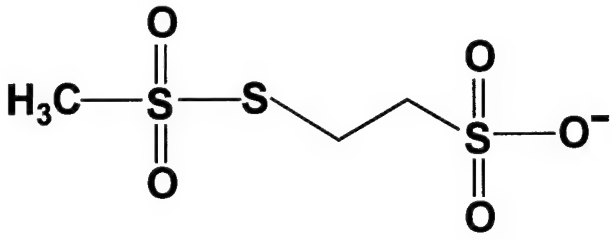
MTSET

[2-(trimethylammonium)ethyl] methanethiosulfonate



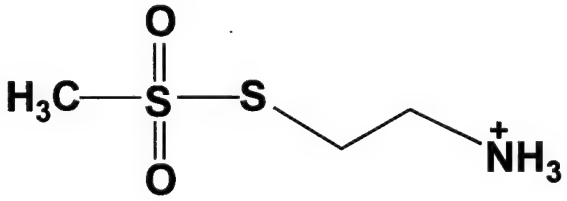
MTSES

(2-sulfonatoethyl) methanethiosulfonate



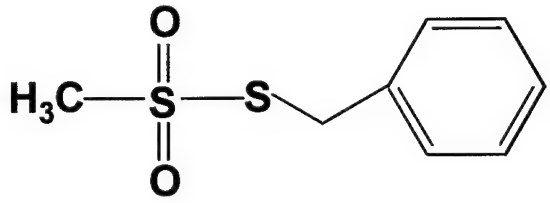
MTSEA

2-aminoethyl methanethiosulfonate



BnMTS

Benzyl methanethiosulfonate



Acrylodan

6-acryloyl-2-dimethylaminonaphthalene

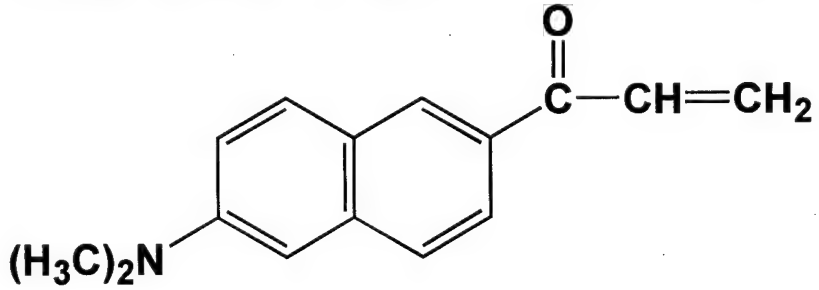
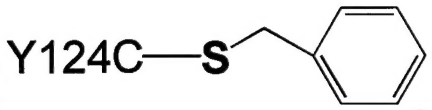
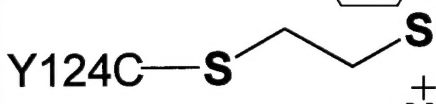
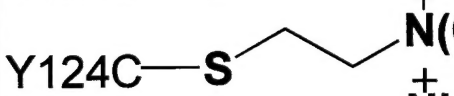
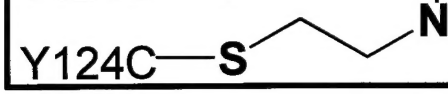
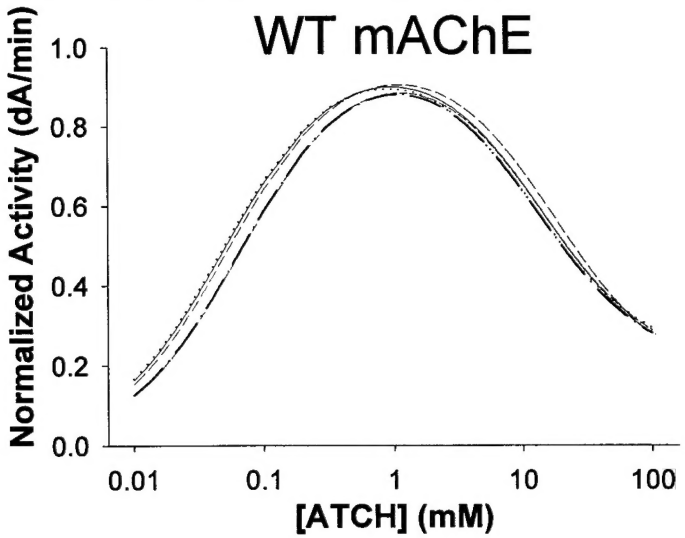
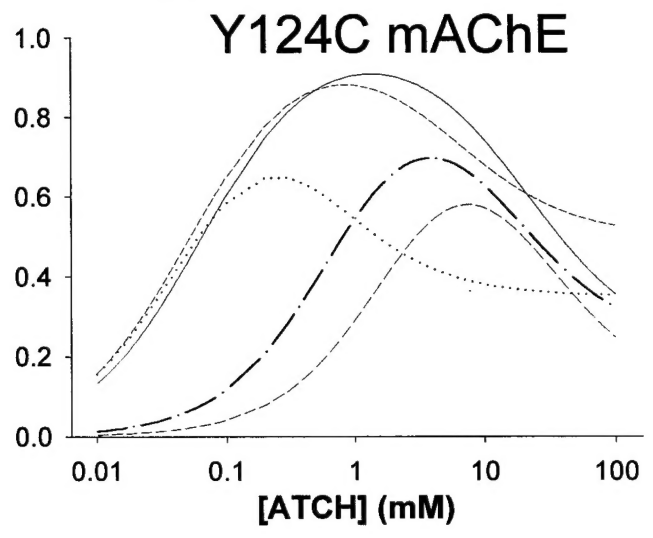
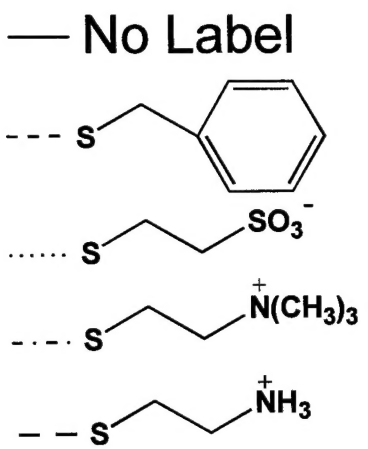


Figure 7

ENZYME	K_m (μM)	k_{cat} (10^5 min^{-1})	$\frac{k_{cat}}{K_m}$ (M^{-1} min^{-1})	$\frac{k_{cat}/K_m\ WT}{k_{cat}/K_m\ Mut}$
WT	51.0 ± 17.1	1.63 ± 0.536	3.23×10^9	1
Y124C	64.9 ± 17.8	1.32 ± 0.320	2.17×10^9	1.49
Y124C—S 	52.7 ± 17.7	ND	ND	ND
Y124C—S—SO_3^- 	53.4 ± 8.92	ND	ND	ND
Y124C—S—$N^+(CH_3)_3$ 	744 ± 8.49	ND	ND	ND
Y124C—S—NH_3^+ 	2300 ± 779	2.03 ± 1.31	0.102×10^9	31.7







Kinetic constants and pS profiles for cysteine substituted and cysteine modified cholinesterases at Tyr 124. Comparison with wild type enzyme.

(7) CONCLUSIONS

The conclusions from the findings are detailed in the five sections. Overall progress has been made in ascertaining orientataion and mechanism of oxime reactivation and developing mutants more susceptible to reactivation. Further resolution of the structure of mammalian AChE, its complexes and conjugates has been achieved. Finally, cysteine substitution mutagenesis shows promise for understanding catalytic mechanism, environment of the gorge, gorge occlusion and charge influences in the gorge. Moreover it provides a route for introducing nucleophiles into the active center.

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